Clinical Proteomics

ISSN 1542-6416/06/02:205–216/$30.00 (Online)

Original Article

Protein Expression Analysis of *Chlamydia pneumoniae* Persistence by Combined Surface-Enhanced Laser Desorption Ionization Time-of-Flight Mass Spectrometry and Two-Dimensional Polyacrylamide Gel Electrophoresis

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Abstract

The aim of this study was to examine the protein expression profiles of persistent *Chlamydia pneumoniae* by two-dimensional polyacrylamide gel electrophoresis (2D PAGE) and surface-enhanced laser desorption ionization time-of-flight mass spectrometry (SELDI-TOF-MS). Although 2D PAGE is still the method of choice for separating and detecting components of complex protein mixtures, it has several distinct disadvantages; i.e., being labor-intensive and having a bias toward proteins within the dynamic range of the gel condition. Hence, SELDI-TOF-MS technology was used to complement 2D PAGE. *C. pneumoniae*-infected HEp2 cells were treated with or without IFN-γ, and protein expression profiles were determined at 48 h postinfection (hpi). Unfractionated monolayers were also used for protein profiling by SELDI-TOF, using two different chip surface types: weak cation exchanger and hydrophobic surface. Under IFN-γ-induced persistence, *C. pneumoniae* expresses an altered protein expression profile. Twenty chlamydial proteins showed differential regulatory patterns

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by SELDI-TOF-MS, two of which, HSP-70 cofactor, and a hypothetical protein, were identified by 2D PAGE and mass spectrometry. Two additional proteins, phosphatidylserine decarboxylase and 30S ribosomal protein S17, were exclusively identified by SELDI TOF-MS analysis, as these were not present in sufficient quantity for detection by 2D PAGE. We propose that a combination of 2D-PAGE and SELDI-TOF-MS may complement the disadvantages of each technique alone and may provide a rapid and precise screening technique.

Key Words: Proteomics; SELDI-TOF-MS; 2D gel electrophoresis; *C. pneumoniae*.

Introduction

Chlamydia pneumoniae is a Gram-negative obligate intracellular pathogen which has a complex biphasic developmental cycle and causes both acute and chronic respiratory infections. Recently, it has been implicated in chronic human disease, specifically atherosclerosis ***(1–5)***. The developmental cycle of Chlamydia initiates when infectious, but metabolically inactive, elementary bodies (EB) enter eukaryotic host cells and differentiate into metabolically active reticulate bodies (RB). The RB then divides by binary fission and subsequently redifferentiates to EB, and are released from the host cell to initiate another lytic cycle ***(6–10)***. In vitro, the chlamydial developmental cycle may be induced into a non-lytic (persistent) phase by IFN-γ (***11***). A subinhibitory concentration of IFN-γ can lead to a state in which redifferentiation of RB to infectious EB is interrupted and the RB develop into an arrested growth stage, commonly known as an aberrant body (AB). Even though it maintains viability while inside the host cell, it is non-culturable and therefore, results in a long-term relationship within the host cells ***(11,12)***.

Although our understanding of Chlamydial persistence at the molecular level is still at its infancy, considerable progress has been made in the recent years. The availability of genome sequences of the chlamydial species ***(13–15)***, as well as the progress made in quantitative RT-PCR, microarray, and proteomics ***(16–24)***, have provided some significant insight into differential expression profiles of the genes/proteins which will help in understanding the common biological properties of this unique pathogen.

We recently reported an analysis of protein expression patterns of *C. pneumoniae* under IFN-γ-induced persistence by employing two-dimensional polyacrylamide gel electrophoresis (2D PAGE), MALDI-TOF-MS and one of the advanced proteomic analysis tools, PDQuest™ software for quantification ***(19)***. This technique is not only labor-intensive, but also limits the isolation of low-abundant, highly hydrophobic membrane proteins, and also those with iso-electric points at either extreme of the pH scale. Moreover, it requires a large amount of protein for analysis, which could be problematic, particularly with clinical samples. Because of difficulties in the study of proteins involved in Chlamydial persistence, as an extension of our previous study, we looked for other high through-put technologies to integrate with existing techniques to better understand the global protein expression profile.

An alternative to 2D PAGE is surface-enhanced laser desorption ionization time-of-flight mass spectrometry (SELDI-TOF-MS), which involves analysis of protein mixtures without the need for an initial protein separation process. This proteomic approach combines the strategy of bio-affinity or chromatographic chemistry with mass spectrometry to identify the selectively captured proteins. It provides spectra of differentially expressed proteins, comparison of which could directly classify the

cellular protein content. In recent years, this technology has been widely used in rapid screening for a large number of potential biomarkers in oncology ***(25,26)***, clinical diagnosis ***(27)***, and infectious diseases ***(28)***. With its superior sensitivity, SELDI-TOF has been proven as a valuable approach for microorganism protein profiling with limited material samples ***(29)***.

In this study, we have integrated the techniques of 2D PAGE with SELDI-TOF-MS to assess the utility of a combined approach for analysis of differential protein expression profiles in *C. pneumoniae*. The rational for this approach is that a combination of SELDI-TOF-MS and 2D PAGE analysis would result in each method complementing each other's strengths. For example, 2D PAGE allows for simultaneous estimation of molecular weight and iso-electric point of a given protein. SELDI-TOF-MS, on the other hand, has a greater dynamic range and molecular weight estimations are more precise, with greater sensitivity, over a given range with incorporation of appropriate calibration.

Methods

Cell Line

HEp2 cells (ATCC CCL-23) were obtained from the American Type Culture Collection (Rockville, MD) and maintained in Iscove's maintenance medium (IMM) (Cellgro, Washington, DC) supplemented with 10% (v/v) fetal bovine serum, 2 m*M* L-glutamine, 1% (v/v) nonessential amino acids, 10 m*M* HEPES buffer, 10 μg/mL gentamycin, 25 μg/mL of vancomycin. Cells were grown in 75-cm^2 flasks (Costar, Cambridge, MA) at 37°C in 5% CO_2 for 24 h to achieve confluency of the monolayer, and harvested with Trypsin-EDTA.

Bacterial Isolate

C. pneumoniae A-03 (ATCC VR-1452) was previously isolated in our laboratory from an atheroma of a patient with coronary artery disease during heart transplantation at the Jewish Hospital Heart and Lung Institute, Louisville, KY ***(1)***. *C. pneumoniae* were propagated in HEp2 cell monolayer in Iscove's growth medium (IGM) as described previously ***(19)***. EBs were harvested and purified by disruption of HEp2 cell monolayers with a cell scraper, sonication, and centrifugation over a renograffin density gradient ***(30)***. EB suspensions were stored in sucrose-phosphate-glutamic acid buffer at –80°C.

Infection Protocol

Similar to our previous study ***(19)***, HEp-2 cells were seeded in six-well plates at 0.5×10^6 cells per well in IMM and incubated in 5% CO_2, at 37°C overnight. Cells were subsequently inoculated with 0.5×10^8 IFU per well of *C. pneumoniae* in 2 mL of Iscove's growth media with or without human recombinant IFN-γ 50 U and 100 U per milliliter (Promega, Madison, WI), centrifuged at 675*g* for 1 h at 10°C, and incubated at 37°C in 5% CO_2 for 24 and 48 h. For proteomic analysis, Chlamydia-infected HEp-2 cells were pulse-labeled for 2 h (at 22 and 46 hpi, respectively) in methionine/cysteine-free RPMI 1640 medium (Cellgro, Herndon, VA) containing 100 μCi of [^{35}S] methionine/cysteine per milliliter (Redivue Pro-mix, Amersham Pharmacia, Piscataway, NJ) and 500 μg of cycloheximide per milliliter in the presence or absence of IFN-γ. At the end of the 2-h labeling period, Chlamydia-infected HEp-2 cells were washed in cold phosphate-buffered saline, scraped with a cell scraper, and pelleted by centrifugation at 16,000*g*.

Protein Extraction

C. pneumoniae-infected HEp-2 cell pellets were resuspended in 30 μL of buffer containing 2% (w/v) sarkosyl, 1% (w/v) OBG (Octyl β-D-1-thioglucopyranoside), 2 m*M* tri-butyl phosphine (TBP), and 5 μL protease inhibitors (leupeptin and aprotinin). Samples were sonicated (Cell dismembrator, Model 100 [Fisher

Scientific]) for 5 s, boiled for 5 min, then cooled to room temperature. One hundred microliters of thiourea lysis buffer (7 *M* urea, 2 *M* thiourea, 10 m*M* Tris, and 2 m*M* TBP and 2% [v/v] ampholine) was added, followed by the addition of 3.0 µL of a mixture of 50 m*M* $MgCl_2$, 476 m*M* Tris-HCl, 24 m*M* Tris base, 1.0 mg of DNase-1 per milliliter, and 0.250 mg of RNase A per milliliter (pH 8.0), incubated on ice for 10 min and stored at –80°C.

2D Gel Electrophoresis

Radio-labeled protein extracts were mixed with IPG (immobilized pH gradient) rehydration buffer containing 8 *M* urea, 4% (w/v) CHAPS, 0.04 *M* Tris Base, 0.065 *M* DTT (dithiothreitol), 0.01% (w/v) bromophenol blue in a final volume of 400 µL as follows. (1) To generate an electrophoretic map of purified *C. pneumoniae* EBs, 750 µg of protein extracts was mixed with IPG rehydration buffer. (2) To study intracellular *C. pneumoniae* protein expression, 75 µg of [^{35}S]-labeled chlamydial proteins was mixed in IPG rehydration buffer. Protein samples were loaded onto Immobiline™ DryStrip (IPG strips 4–7, 6–11, or 3–10) (Amersham Biosciences, Uppsala, Sweden), allowed to rehydrate overnight and isoelectrofocused to 98,000 V hours in PROTEAN® IEF cell (Bio-Rad). The following focusing parameters were applied: 7000 V of maximum voltage, 500 V of holding voltage, 50 µA of maximum current per gel, and for a duration of 16 h. After focusing was completed, IPG strips were equilibrated in buffer containing 6 *M* urea, 2% (w/v) DTT, 30% (v/v) glycerol, and 1X Tris-acetate. The second dimension was carried out using 10% polyacrylamide, 20 × 20-cm slab gels in PROTEAN II xi multi-cell system (Bio-Rad) at 4°C for 4.5 h under a 500-V maximum voltage and 20 W per gel, with 200 m*M* Tricine used as a cathode buffer, and 0.4% (w/v) SDS plus 625 m*M* Tris-acetate (pH 8.3) used as an anode buffer. Gels were fixed in 40% ethanol –7.5% acetic acid for 30 min. Gels containing purified EB proteins were silver-stained, whereas gels containing radio-labeled chlamydial proteins were treated for 30 min with Amplify fluorographic reagent (Amersham Pharmacia, Buckinghamshire, England), vacuum dried, and exposed to high density phosphor-imaging screens (Bio-Rad) for 2.5 d. Images were scanned in a Molecular Imager® FX Pro Plus™ system (Bio-Rad). This imaging equipment is fully integrated to the ProteomeWorks™ system (Bio-Rad) with a high-resolution image acquisition interface within the PDQuest software.

Image Analysis and Identification of Protein Spots

Protein spots were analyzed and quantified for differential expression patterns between gels treated with and without IFN-γ using PDQuest software v7.1 (Bio-Rad) as explained previously ***(19)***. Briefly, PDQuest software v7.1 interfaced directly with the phosphor-imager using ProteomeWorks software. Protein spots on the untreated and IFN-γ treated gels were aligned by the pattern recognition feature of the software. During spot detection, the original scanned gel image is filtered to remove background noise, and the filtered images are used as the basis for Gaussian modeling for quantitative purposes. PDQuest software was also used for quantification, in which pixel values for each identified spot were first normalized as a percentage of total pixel quantity of all valid spots for each individual gel image. After normalization, analytical tools in PDQuest software were used for statistical analysis of differential expression patterns that allow spots to be grouped under user-defined fold increase or decrease regulatory changes.

Protein spots from *C. pneumoniae* EBs were excised from silver-stained gels and treated with 20 µg Trypsin per milliliter in 50 m*M* ammonium bicarbonate at 37°C overnight. Two microliters of supernatant was mixed in

an equal volume of saturated α-cyano-4-hydroxycinnamic acid, 50% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid (TFA), and 0.8 μL of the resulting solution was applied to the MALDI-MS template. Masses of peptide fragments were determined by MALDI–time-of-flight (TOF) analysis with a Micromass mass spectrometer. Patterns of measured masses were matched against theoretical masses of proteins found in the annotated databases SWISS-PROT and TREMBL, accessible in the ExPASy Molecular Biology server (http://expasy.cbr.nrc.ca/). Searches were performed with the profound-peptide mapping (The Rockefeller University Edition v4.10.5) with restrictions to proteins from 1 to 100 KD and mass tolerances of 100 ppm. Partial enzymatic cleavages leaving one cleavage site, oxidation of methionine, and modification of cysteine with iodoacetamide were considered in these searches.

SELDI-TOF-MS Analysis

Weak cation exchange and hydrophobic surface ProteinChip arrays were preequilibrated in the binding/washing buffers of either 100 m*M* sodium acetate (pH 4.0) with 0.1% Triton X-100 for cation exchange, or 50 m*M* HEPES (pH 7.0) with 0.1% Triton X-100 for hydrophobic surface. Protein extracts (25 μg per spot) were prepared by vortexing in a buffer containing 9 *M* urea, 2% CHAPS and 50 m*M* Tris (pH 9.0) at 4°C for 30 min. The samples were then diluted 40-fold with the corresponding binding/washing buffers and applied to the ProteinChip arrays assembled in a bio-processor. A 96-well formatted bio-processor reservoir system, allowing multi-chip processing of proteins in several samples simultaneously, was sealed and vigorously agitated at room temperature for 30 min. This allows the proteins to bind to the chip surface either through charge interaction or by reversed phase hydrophobic interaction. The unbound proteins were washed away with the corresponding binding/washing buffers for three times. The arrays were rinsed with 1 m*M* HEPES (pH 7.0) twice before removal from the bio-processor. After air-drying, two additions of 1 μL of saturated sinapinic acid were applied to each spot of the arrays. The ProteinChip was analyzed on the Protein Biological System (PBS-II) mass spectrometer (Ciphergen). The data was automatically collected by two sequential acquisitions, in which, one was for the low mass range from 5 to 30 KD with a laser intensity of 220 mJ, and another with a laser intensity of 250 to focus on the high mass range from 30 to 80 KD. The external calibration equation, all-in-1 protein molecular weight standard (Ciphergen), was applied to all spectra for calibrating the mass accuracy. The obtained data was analyzed by Biomarker Wizard software from Ciphergen.

Bioinformatics

Upregulated or unchanged protein peaks, which were discovered by SELDI-TOF-MS analysis, were identified by bioinformatics approach using TagIdent tool (http://us.expasy.org/tools/tagident.html). The molecular weight (mw) and pI of such spots corresponding to these protein peaks were also estimated from the analytical 2D PAGE, and applied to TagIdent. Very stringent parameters and specified error margins of a pI range of ±0.25 and mw range of ±10% were used to match proteins from the database (Swiss-Prot/TrEMBL/NCBI) with our observed mw and pI (p-value of <0.05).

Results and Discussion

The primary objective of this study was to integrate the two techniques of 2D PAGE and SELDI-TOF-MS to further examine differential expression patterns of *C. pneumoniae* under IFN-γ-induced persistence. In order to assess the validity of comparative analysis by 2D PAGE and SELDI-TOF-MS, the accuracy of each technique was established with known molecular weight standards **(Table 1)**. The molecular weights of the seven protein

Table 1
Assessment of Standard Protein Markers Used for SELDI-TOF-MS and 2D PAGE

	Theoretical values		SELDI analysis	2D PAGE	
Protein standards	Mol. wt.	pI	Mol. wt.	Mol. wt.	pI
Hirudin BHVK ***(36)***	7034	Varies	7475	7000	7.6–7.8
Cytochrome C (Bovine) ***(37)***	12,230	10.37–10.8	11,550	12,000	7.6–8.2
Myoglobin (Equine) ***(38)***	16,951	7.3 major comp. 6.8 minor comp.	17,134	19,000	8.0–8.4
Carbonic anhydrase (Bovine RBC) ***(39)***	29,023	5.9	28,988	31,000	5.5–9.0
Enolase *(S. cervisiae)* ***(40)***	46,671	6.1	46,531	50,000	6.4–8.3
Albumin (bovine) ***(41)***	66,433	4.7–4.9	66,379	75,000	4.0–6.2
IgG (Bovine) (Sigma, cat. no I5506)	147,300	Heterogenous	147,034	150,000	Smear

standards (e.g., hirudin, cytochrome C, myoglobin, carbonic anhydrase, enolase, albumin, and IgG) were measured by SELDI-TOF-MS as well as 2D PAGE. Additionally, the iso-electric points of all the protein standards were determined by 2D PAGE. The molecular weights and iso-electric points of all seven protein standards showed considerable correlation when compared with each of the two techniques, indicating good concordance of SELDI-TOF-MS and 2D PAGE techniques.

Using SELDI-TOF-MS, a total of 1751 proteins were detected in the three types of monolayers analyzed: *C. pneumoniae*-infected HEp2 monolayers with or without IFN-γ and uninfected HEp2 monolayers with IFN-γ, as a control. All monolayers were analyzed by both hydrophobic and weak cation exchange chip types. Twenty *C. pneumoniae* proteins, which were differentially expressed and showed a *p*-value of <0.04, were further analyzed by the combination of both the methods **(Table 2)**. With respect to proteins found by SELDI TOF-MS analysis, proteins within the same range of molecular weight were located in the 2D PAGE. Of the 20 proteins, we confirmed the identity of 2 *C. pneumoniae* proteins expressed intracellularly at 48 hpi under infectious (IFN-γ untreated) as well as IFN-γ treated (100 U/mL) by MALDI-TOF-MS. These two proteins, CPn0710 and GrpE, are depicted in bold in **Fig. 1** and **Table 1**. Both of these proteins had *p*-value of <0.01, and showed differential regulatory pattern when induced with IFN-γ. Consistent with in our previous study ***(19)***, CPn0710 was found to be 35.4-fold downregulated, whereas, GrpE was upregulated by 1.6-fold. The regulatory pattern of these two proteins, when analyzed by SELDI-TOF-MS, showed CPn0710 to be 1.5-fold downregulated and GrpE 1.4 upregulated, indicating substantial agreement between the two techniques.

Although function of the CPn0710, a hypothetical protein, is still unknown, GrpE protein stimulates, jointly with DnaJ, the ATPase activity of the DnaK chaperone ***(31,32)***. It accelerates the release of ADP from DnaK thus allowing DnaK to recycle more efficiently and therefore may suggest its upregulation during IFN-induced stress condition in *C. pneumoniae.*

For the other 18 proteins, a bioinformatics approach was implemented by using the TagIdent tool to locate the protein on the 2D gel. Briefly, the information obtained on m/z ratio by SELDI-TOF-MS analysis and the corresponding pI of the spots in the 2D gel

Table 2
Proteins Discovered by 2D PAGE Analysis With Respect to SELDI-TOF-MS Quantification

Proteins found by SELDI-TOF-MS analysis only				Proteins found by 2D PAGE with respect to SELDI-TOF-MS			
Probable protein	Function	Mol. wt.	pI (theoretical)	Probable protein	Function	Mol. wt.	pI
PsdD	Phosphatidylserine decarboxylase	5070.88	7.94		No protein found by 2D PAGE		
InfA	Translational initiation factor IF-1	8354.94	9.36	XseB	Exodeoxyribonuclease VII	8000	4.2
CPn0710	**Hypothetical protein**	**9347.8**	**5.1**	**CPn0710**	**Hypothetical protein**	**9500**	**4.8**
Rs18	30 S ribosomal protein S18	9533.15	10.8	CPn0711	Hypothetical protein	9400	6.3
Rs17	30 S ribosomal protein S17	9888.7	10.41		No protein found by 2D PAGE		
CPn0818	Putative outer membrane protein	10,550.1	7.95	CPn0039	Hypothetical protein	10,500	5.2
CPn0039	Hypothetical protein	10,819.6	4.99	CPn0039	Hypothetical protein	10,500	5.2
CPn0592	Hypothetical protein	11,750.8	7.63	TrxA	Thioredoxin	11,500	5.0
Rl22	50 S ribosomal protein L22	12,428.5	10.8	Rs6	30 S ribosomal protein S6	12,000	6.5
GcvH	Glycine cleavage system H	12,875.7	4.38	GcvH	Glycine cleavage system H	12,000	4.4
Rl18	50 S ribosomal protein L18	13,636.1	10.51	Rl7	50 S ribosomal protein L7/L22	13,500	4.9
CPn0713	Hypothetical protein	14,173.1	4.52	CPn0713	Hypothetical protein	14,000	4.5
Dut	dUTP pyrophosphate	15,346.5	5.0	Dut	dUTP pyrophosphate	15,000	5.0
GrpE	**HSP-70 cofactor**	**21,291**	**4.88**	**GrpE**	**HSP-70 cofactor**	**21,000**	**4.7**
RibE	Riboflavin synthase alpha chain	21,700.9	4.94	RibE	Riboflavin synthase alpha chain	21,500	4.9
CPn0206	Hypothetical protein	28,236.4	4.46	CPn0206	Hypothetical protein	28,000	4.6
Pgl	6-phophoglucanolactonase	29,333.5	4.92	Pgl	6-phophoglucanolactonase	29,500	4.9
CPn0087	Hypothetical protein	31,322.3	4.7	CPn0087	Hypothetical protein	31,000	4.7
DnaA_1	Replication initiator protein	52,940.8	6.45	Pyk	Pyruvate kinase	53,000	5.5
Pmp_12	Polymorphic membrane protein	53,876.04	4.94	Pmp_12	Polymorphic membrane protein	53,500	4.8

The proteins in bold were identified by both 2D PAGE-MALDI-TOF-MS as well as SELDI-TOF-MS. The highlighted proteins were identified by SELDI-TOF-MS only.

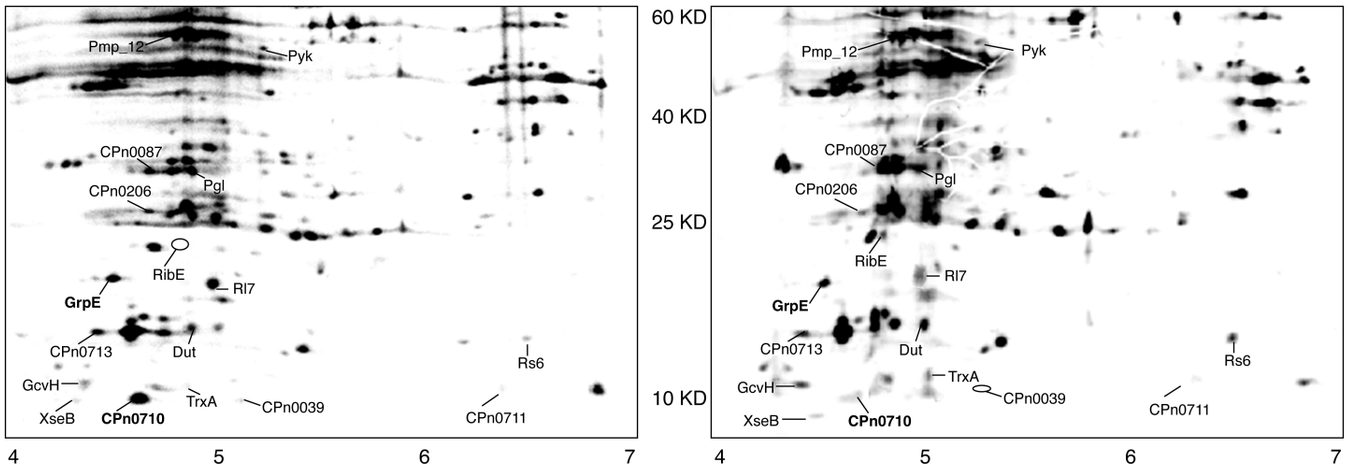

Fig. 1. Representative two-dimensional electrophoretic map of *Chlamydia pneumoniae* proteins expressed at 48 hpi in HEp2 cells in the absence (untreated) and presence (IFN-treated) of 100 U IFN-γ/mL. The proteins labeled in bold were identified by both 2D PAGE-MALDI-TOF-MS as well as SELDI-TOF-MS.

(Table 1; **Fig. 1)**, provided the data for identifying the protein spots. Interestingly, out of these 18 proteins, 2 of the proteins, PsdD and Rs17, were discovered exclusively by SELDI-TOF-MS analysis (highlighted in **Table 1**), and which could not be resolved by 2D PAGE. Both proteins showed a *p*-value of 0.02. The fact that PsdD, a 5-kD protein, and Rs17, a highly alkaline protein (pI = 10.41), were detected exclusively by SELDI-TOF-MS analysis, further confirms our assumption that coupling of these two techniques offers a distinct advantage, because proteins that are in low abundance, low Mw, and with extreme pI, can be very difficult to detect by 2D PAGE, whereas, can be readily detected by SELDI-TOF-MS.

Rs17 is a 30S ribosomal protein which is involved in the translation process. PsdD converts phosphatidylserine to phosphatidylethanolamine during phospholipid biosynthesis in bacteria ***(33*,*34)***. Phospholipids are major structural components of cell membranes and also play an important role in membrane-localized processes. Inhibition of PsdD alters the transport activities inside the bacterium because of altered phospholipids composition, which not only affects phospholipid synthesis, but also, other cellular activities ***(35)***. PsdD was found remarkably upregulated in the IFN-treated cultures. This perhaps indicates that *C. pneumoniae* allows influx of ions across the membrane and keeps a basal level of metabolism going to remain viable under hostile condition.

Taken together, we have shown that SELDI-TOF-MS is a high throughput screening tool with high sensitivity, whereas 2D PAGE is an excellent discovery tool, which allows estimation of both Mw and pI of a given protein and also helps visualizing isoforms of certain proteins. Although there is considerable correlation between the two techniques, there are some disagreements in the bioinformatics identification. 2D PAGE is likely to be more accurate, as it utilizes Mw and pI and perhaps may be better for quantification of regulatory patterns.

We tested the combination of these two techniques to study one model of *C. pneumoniae* persistence. Such a combined approach could be used in other systems as well, and may be a valuable tool in directly measuring the protein levels and activity within the host cell. Additionally, it may also play a role in mapping differential protein expression profiles between healthy and diseased individuals, which may lead to discovery of biomarkers specific for that disease process.

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